THE EFFECT OF NITROGEN MUSTARDS ON THE RESPIRATION AND FERTILIZATION OF SEA URCHIN SPERM AND EGGS [1]

E. S. GUZMAN BARRON, J. E. SEEGMILLER, E. G. MENDES, AND H. T. NARAHARA

Chemical Division, Department of Medicine, The University of Chicago, and the Marine Biological Laboratory, Woods Hole

The halogenated alkyl amines, the so-called nitrogen mustards, are extremely interesting compounds to the biologist. When dissolved in water they give quaternary nitrogen transformation products which in small amounts inhibit mitosis (Friedenwald and Schultz, 1943; Bodenstein, 1947) and produce mutations (Auerbach *et al.*, 1943; Miller and McElroy, 1948). These transformation products act as structural inhibitors for those enzymes where choline is the substrate in the enzyme-substrate complex. Furthermore, nitrogen mustards act as inhibitors of other enzymes by virtue of the great reactivity of their ethylene immonium derivatives and of their halogen groups. All these enzyme inhibitions produce inhibition of tissue respiration and of synthesis reactions (Barron, Bartlett and Miller, In press; Barron, Bartlett, Miller, Meyer and Seegmiller, In press). The effect of nitrogen mustards on cell division of fertilized sea urchin eggs was studied by Cannan and Levy (1944) and Vislocki *et al.* (1945), who found that they produced a delay in the rate of cellular division. As continuation of the work of this laboratory on the mechanism of action of nitrogen mustards, the effect of these compounds on the respiration and fertilization of sea urchin eggs and sperm was studied. Nitrogen mustards were found to produce in sea urchin sperm a striking stimulation of respiration accompanied by an inhibition on fertilization of eggs of the normal sequences of cell division and development.

Experimental

Male and female species of *Arbacia punctulata* were allowed to shed their sperm and eggs. The eggs were washed in a large amount of filtered sea water and the excess water was withdrawn previous to the experiments. The sperm were suspended in twelve volumes of filtered sea water, shaken, centrifuged for five minutes, and resuspended in the desired amount of sea water. The nitrogen mustards (their HCl salts) were dissolved in sea water just before use and were added to the sperm suspension 15 to 30 minutes previous to the measurement of respiration. The manometric experiments were performed at 25° C.; the fertilization experiments, at the room temperature of the laboratory, 23°–25° C.

Effect of nitrogen mustards on the respiration of sperm and of eggs of sea urchin. Freshly dissolved methyl bis (β-chloroethyl) amine HCl (MBA) at a concentration of 0.001 *M* added to sperm suspensions from 15 to 30 minutes be-

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TABLE I

Effect of methyl bis (β-chloroethyl) amine HCl (MBA) on the respiration of sea urchin sperm

The cells were suspended in sea water. Temperature 25° C.

Experiment No.	O_2 Uptake per Hour		
	Control	MBA	Increase
	c. mm.	c. mm.	Per cent
I	46.6	128.8	176
II	56.5	139	146
III	80	115	44
IV	41	84	105

fore measurement of the O_2 uptake increased it from 44 to 176 per cent (Table I). This variation in the increase of respiration is probably due to the different periods of times which elapsed from addition of MBA to the measurement of the O_2 uptake. In fact, when MBA was added while respiration was being measured, there was an induction period of about 30 minutes when the O_2 uptake continued at the same rate as before addition of MBA, the increase starting afterwards (Fig. 1). This induction period must be due to the slow formation in sea water of the active ethylene immonium transformation product (transformation of nitrogen mustards into the ethylene immonium derivative is greatly retarded in the presence of KCl and NaCl).

Increase in respiration was also shown on addition of other halogenated alkyl amines. In experiments performed under similar conditions, MBA increased the respiration of sea urchin sperm by 196 per cent; isopropyl bis (β-chloroethyl) amine HCl, 115 per cent; ethyl bis (β-chloroethyl) amine HCl, 155 per cent; and tris (β-chloroethyl) amine HCl, 79 per cent (Table II).

The increase in respiration was obtained with concentrations varying between 0.001 *M* and 0.0001 *M*. When the concentration of MBA was diminished to 0.00001 *M* there was no effect at all with sperm suspensions of 1:20. With concentrations of MBA of 0.0001 *M*, the increase in respiration, which did not start until one hour and a half after addition, remained constant for the duration of the experiment, eight hours. However, when the concentration was increased ten

TABLE II

The effect of nitrogen mustards on the respiration of sea urchin sperm

The nitrogen mustards (0.0001 *M*) were dissolved in sea water and added 20 minutes before measurement of the O_2 uptake.

Nitrogen mustard	O_2 uptake	
	Control	N Mustard
	c. mm.	c. mm.
Methyl bis (β-chloroethyl) amine HCl	42	124
Isopropyl bis (β-chloroethyl) amine HCl	42	90
Ethyl bis (β-chloroethyl) amine HCl	42	107
Tris (β-chloroethyl) amine HCl	42	75

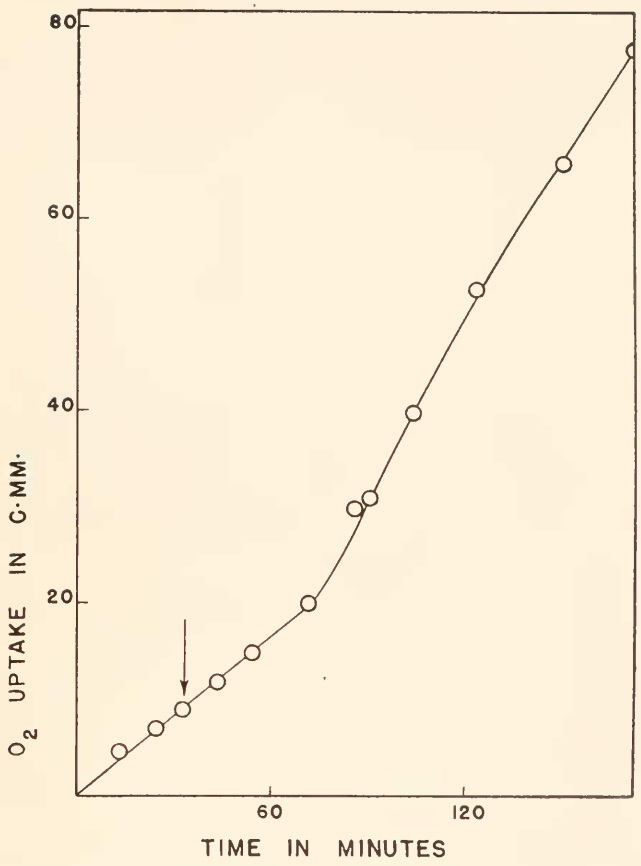

FIGURE 1. Effect of methyl bis (β-chloroethyl) amine HCl (0.001 *M*) on the respiration of sea urchin sperm. MBA was added at time marked by arrow. Temperature 25° C.

times (0.001 *M*) the increase in respiration diminished after three hours (Fig. 2). This increase of respiration followed by a diminution seemed to indicate that increase and inhibition could be obtained by either increasing the concentration of nitrogen mustard or by decreasing the concentration of sperm cells. The latter method was chosen to test this possibility. Sperm suspensions were diluted with sea water as follows: 1 of sperm and 10 of sea water; 1:50; 1:100 and 1:200. After addition of 0.001 *M* MBA, the O_2 uptake of the sperm suspensions was measured for nine hours. The most striking results were obtained on sperm suspensions diluted 1:200. In the first hour, MBA increased the respiration by 120 per cent; in the second hour this increase dropped to 54 per cent; and in the third hour it disappeared completely. In the fourth hour the inhibition started (27 per cent), to become complete in the sixth hour (Fig. 3). Sperm suspensions at this dilution had a remarkably high respiration, for the Q_{o_2} of the control samples was 26.0; furthermore, the O_2 uptake remained at steady values throughout the duration of the experiments, nine hours.

TABLE III

Effect of MBA (0.001 M) on the respiratory quotient of sea urchin sperm

Experiment No.	O_2 uptake		CO_2 production	
	Control	MBA	Control	MBA
I	54.5	171	50	147.5
II	62.0	164	70	140
III	76	137	75	128
IV	49	147	50	144

This increase seems to be confined to oxidative processes because nitrogen mustards had no effect on the anaerobic glycolysis of sea urchin sperm.

The increase in respiration seems to take place on the whole respiratory process as shown by measurements of the respiratory quotient (R.Q.). The R.Q. of sea urchin sperm is close to 1 as found by Barron and Goldinger (1941). The average R.Q. value of four experiments (triplicate determinations for each experiment) was 1.01 for the control samples. The R.Q. of the nitrogen mustard containing samples was 0.93 (Table III).

The respiration of sea urchin eggs whether fertilized or unfertilized was not affected by addition of MBA or isopropyl bis (β-chloroethyl) amine HCl (Table IV). This lack of effect cannot be due to lack of penetration for nitrogen mustards added to sea urchin eggs produce a delay in the cleavage of eggs when they are fertilized with normal sperm (Cannan and Levy, 1944; Vislocki *et al.*, 1945).

Inhibitions of enzymes and of tissue respiration produced by nitrogen mustards can be prevented on addition of thiosulfate or of choline (Barron, Bartlett and Miller, In press). Previous addition of these two substances to sperm at a concentration 50 times that of MBA had no effect at all on the increased respiration produced by the nitrogen mustard. The increase in respiration was not associated with any possible combination of seminal fluid with the nitrogen mustard (if we accept the postulated inhibition of respiration by seminal fluid), for stimulation of

TABLE IV

Effect of MBA and isopropyl bis (β-chloroethyl) amine HCl on the respiration of sea urchin eggs

Nitrogen mustard (0.001 *M*)	O_2 uptake in 1 hour			
	Unfertilized eggs		Fertilized eggs	
	Control	N. mustard	Control	N. mustard
MBA	19.1	16.8	40	37
MBA	17.0	15.0	46	44
Isopropyl bis (β-chloroethyl) amine HCl	20	18	52	54
Isopropyl bis (β-chloroethyl) amine HCl	21	23	32.5	31.5

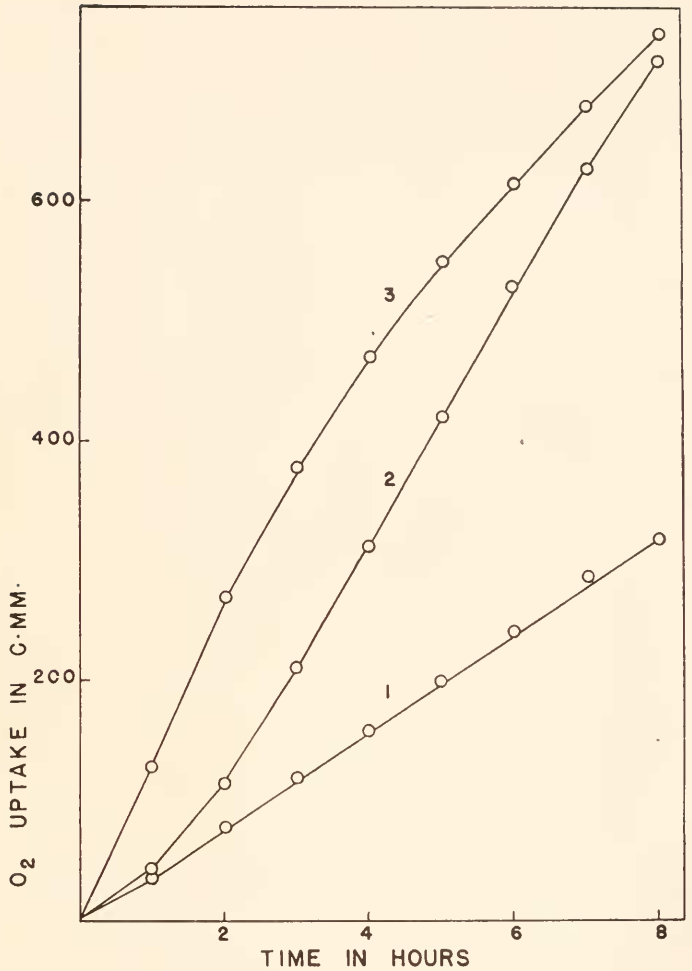

FIGURE 2. Increase in respiration produced by MBA on sea urchin sperm. Effect of concentration of MBA. 1. Control; 2. MBA, 1×10^{-4} *M*; 3. MBA, 1×10^{-3} *M*.

respiration was obtained on sperm suspensions washed repeatedly with sea water by successive suspensions and centrifugation.

Effect of MBA on the fertilization of Arbacia eggs. Cannan and Levy (1944) and Vislocki *et al.* (1945) found that nitrogen mustards in small concentrations produced a delay in the rate of cleavage of sea urchin eggs. In their experiments 0.0013 *M* MBA was added to a suspension of eggs, and 20 minutes later the eggs were washed and fertilized with non-treated sperm. Under those conditions, while first cleavage (50 per cent) took place in 56 minutes in the control eggs, it required 78 minutes in the MBA treated eggs. Treatment just before fertilization started retarded first division but not the second division. The inhibition of cell division was found more effective when the sperm was treated with nitrogen mustard, as can be seen in the two series of experiments.

First series of experiments. A suspension of sperm (1:50) was added to different concentrations of MBA to give the following concentrations: 1×10^{-3} *M*; 1×10^{-4} *M*; 1×10^{-5} *M*; and 1×10^{-6} *M*. Forty minutes later, 0.1 cc. of this sperm suspension was added to 20 cc. of sea water containing unfertilized eggs. During the first cleavage period the eggs receiving sperm suspensions with an initial MBA concentration of 1×10^{-3} *M* and 1×10^{-4} *M* (concentration in the egg suspension, 5×10^{-5} and 5×10^{-6}) showed only 1 and 3 per cent of eggs in the two-cell stage. The eggs receiving sperm with an initial MBA concentration of 1×10^{-5} *M* and 1×10^{-6} *M* showed 75 per cent and 92 per cent cells in the two-cell stage. Thirty hours later, when the control cells were all motile, in the blastula and pluteus stages, the cells receiving sperm with MBA at the initial concentrations of 1×10^{-3} *M* and 1×10^{-4} *M* were all nonmotile; the cells receiving sperm with MBA concentration of 1×10^{-5} *M* had 30 per cent of motile cells and those receiving sperm with 1×10^{-6} *M* MBA had 50 per cent of motile cells. Furthermore a number of morphological alterations were observed (Table V).

TABLE V

Effect of MBA added to sperm on the fertilization and development of sea urchin eggs
The sperm was diluted 1:200.

Time after fertilization	Control	Initial concentration of MBA, *M*			
		1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
Minutes	Per cent	Per cent	Per cent	Per cent	Per cent
66—First cleavage					
2 cell stage	80	1	3	75	92
118—Second cleavage					
2 cell stage	18	65	85	20	18
4 cell stage	75	None	3	74	82
180—2 cell stage	—	4	2	2	
4 cell stage	10	39	7	3	2
Many cell stage	90	37	87	95	98
25 hours—Blastula Motile	60	3	20	75	60
30½ hours—Motile Blastula	All	None	None	30	50

Second series of experiments. Suspensions of sperm were treated with 1×10^{-3} *M* MBA and twenty minutes later 0.05 cc. of this suspension was added to a suspension of eggs in 5 cc. sea water. A suspension of unfertilized eggs was treated with an amount of MBA corresponding to that received by the eggs fertilized with MBA treated sperm; twenty minutes later they received normal sperm. At the end of 158 minutes, while the control and the treated eggs had 90 per cent of cells in the 4-cell stage, the eggs receiving treated sperm had only 39 per cent. Twenty-seven hours later the control and the MBA treated eggs had the same amount of

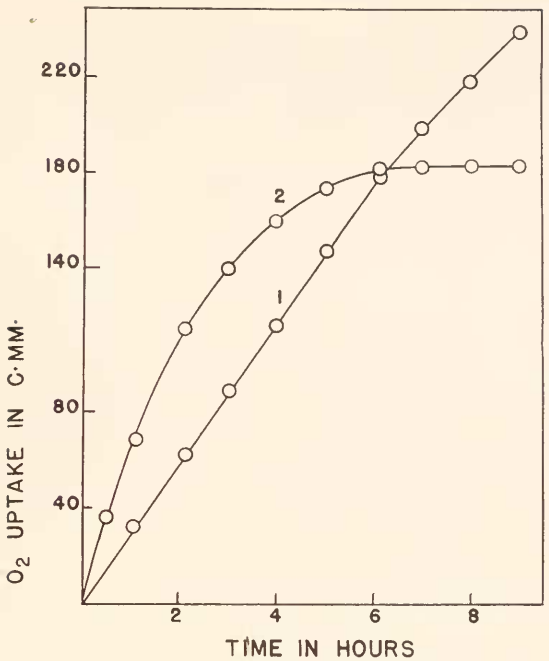

FIGURE 3. The effect of MBA (0.001 *M*) on the respiration of dilute sperm suspensions. Dilution, 1:200. $Q_{O_2} = 26.0$; 1. Control; 2. MBA.

actively moving pluteus (45 per cent) while the eggs receiving treated sperm had only 8 per cent of motile pluteus and had 16 per cent of cells morphologically abnormal.

Delay in the rate of cell cleavage, decrease in the number of cells reaching the pluteus stage, as well as abnormal cells, were found even when sperm and MBA were in contact for only two minutes before fertilization.

DISCUSSION

The sperm of sea urchin is a striking exception in its response to the addition of nitrogen mustards. While other cells or tissues are either inhibited or not affected in their respiration, the sperm of sea urchin showed a marked increase. This increase, which comprises the whole respiration process there being no alteration in the respiratory quotient, must be due to the action of nitrogen mustards on the regulatory mechanisms of the cell, i.e., those mechanisms which control the equilibrium relationships between anabolic and catabolic processes. The increase in respiration seems to occur, however, only when small concentrations of nitrogen mustard penetrate into the cells, for when the cell concentration was decreased complete inhibition of respiration followed the initial stimulation. Undoubtedly, as more nitrogen mustard penetrated the cells a concentration was reached—enough to produce the known enzyme inhibitions found by Cori (1943), Dixon (1942) and Barron *et al.* (In press). The stimulation of respiration might be due to

combination of the halogen groups of nitrogen mustard with soluble — SH groups in the sperm cell, groups which act as regulators of respiration. Nitrogen mustard produced also a profound impairment in the fertilization power of sperm. In fact, sperm treated with nitrogen mustard was more effective in inhibiting the rate of cell division of fertilized eggs. Furthermore, the eggs were stopped in their development so that none reached the pluteus stage. The mechanism of this inhibition is not yet known; it is undoubtedly related to the property of nitrogen mustards of inhibiting mitosis and of producing mutations.

Summary

The halogenated alkyl amines, methyl bis (β-chloroethyl) amine HCl, isopropyl bis (β-chloroethyl) amine HCl, ethyl bis (β-chloroethyl) amine HCl, and tris (β-chloroethyl) amine HCl, when added to sperm suspensions of *Arabacia punctulata* increased their respiration. This increase seems to affect the entire respiratory process as shown by the unaltered R.Q. value. Anaerobic glycolysis was not altered. With dilute sperm suspensions (1:200) the stimulation of respiration was followed by inhibition. Sperm so treated produced, on fertilization of eggs, an inhibition in the rate of cleavage, of pluteus formation, and motility of blastula, and produced in addition a number of malformations.

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